REMARKS

Claims 1-24 are currently pending in the application. Claim 14 has been canceled, claims 1 and 15 has been amended and new claim 25 has been added. Accordingly, claims 1-13 and claims 15-25 will be pending upon entry of this amendment and response. Support for amended claims 1 and 15 and new claim 25 can be found throughout the application and in the claims as originally filed. Specifically, support for amended claim 1 can be found at least, for example, at page 6, line 2 through page 8, line 1 and page 12, lines 1-12. Support for new claim 25 may be found at least, for example, at page 10, lines 1-7 and page 12, lines 4-12.

No new matter has been added. Any amendments to and/or cancellation of the claims should in no way be construed as acquiescence to any of the rejections and was done solely to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Attached hereto is Appendix A, captioned "Version With Markings To Show Changes Made," indicating the changes made to the claims. For the Examiner's convenience, all of pending claims are set forth in Appendix B.

Rejection of Claim 14 Under 35 U.S.C. §112, Second Paragraph

Claim 14 has been rejected under 35 U.S.C. §112, second paragraph as "being indefinite to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Specifically, claim 14 is indefinite because "it is unclear how the claim further limits or is patentably distinct from its base claim."

Without acquiescing to the Examiner's rejection, claim 14 has been canceled *without prejudice*, thus rendering the rejection moot as it pertains to this claim. Applicants submit that this amendment obviates the rejection, and reconsideration and withdrawal of the rejection is respectfully requested.

Rejection of Claims 1-13 and 20-24 Under 35 U.S.C. §112, First Paragraph

Claims 1-13 and 20-24 were rejected for lack of adequate written description on the ground that the rejected claims embrace a broad range of promoters. Specifically, it is stated in the Office Action that

> ...the only promoters adequately describe in the instant specification are those whose function is suppressed in non-tumor cells, but up-regulated in tumor cells in accordance with a particular p53 or p16 status (see page 3, third paragraph of the Office Action).

and further that

> ...[t]he problem with the instant claims is that [they] are solely defined by functional characteristics that are not coupled with a known or disclosed [sic] correlation between function and structure in accordance with the Interim Guidelines for the Written Description Requirement...[t]he specification does not [disclose] what common structural features in the HSP70, MDR1, PCNA or p16INK4 promoters account for their meeting the requirements of a Type I promoter (see page 4, first full paragraph of the Office Action).

Without acquiescing to the rejection, claim 1 has been amended to clarify the subject matter that Applicants view as the invention. Specifically, claim 1 has now been amended to specify, in pertinent part, "...a first promoter whose function is suppressed *by a wild-type p53 or* $p16^{INK4}$ *allele* in non-tumor cells relative to tumor cells *carrying a mutant p53 or p16 allele...*"

Applicants respectfully submit that claim 1, as amended, together with the specification and claims as originally filed, provides sufficient written description regarding the promoters that fall within the scope of the claims, to inform a skilled artisan that Applicants were in possession of the claimed invention at the time the application was filed, as required by §112, First Paragraph (see M.P.E.P. 2163.02).

As the Examiner is well aware, written description "...may be satisfied through disclosure of relevant identifying characteristics, *i.e.*, structure, other physical and/or chemical characteristics, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." *Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. §112, First Paragraph Written Description Requirement.* Moreover, "[a] specification may, within the meaning of 35 U.S.C. §112, First Paragraph, contain a written description of a broadly written claimed invention without describing all species that claim encompasses." *Utter v. Hiraga*, 845 F.2d 993, 6 USPQ2d 1709 (Fed. Cir. 1988).

Applicants respectfully submit that the claimed genus of promoters of the present invention is adequately defined by functional characteristics when coupled with a known or disclosed correlation between function and structure. The specification teaches that the general concept of the invention may be practiced using genes involved in ***tumor suppressor pathways*** (see, *e.g.*, page 6 lines 2-8). In other words, the genes suitable for Type I promoters would be those that are strongly down regulated in the presence of a wildtype tumor suppressor protein. As specific examples, the specification teaches the use of promoters involved in the p53 and $p16^{INK4}$ tumor suppressor pathways, such as p16, Bcl-2, MDR-1, HSP70, PCNA and CMV (see, *e.g.*, page 6, line 2 through page 8, line 1 and page 12, lines 2-12). The specification also provides working examples including the wildtype p53 and mutant p53 promoters, and discloses examples of other promoters that are expected to work in the invention as claimed, such as the HSP70, MDR1 and PCNA promoters (see, *e.g.*, page 10, lines 1-7). Furthermore, the specification also provides guidance which would allow the skilled artisan to test any promoter to determine whether it has the required characteristics (see, *e.g.*, at pages 14-15, and in the assays disclosed in the examples at pages 28-45). Therefore, reconsideration and withdrawal of the rejection is respectfully requested.

Claims 1-24 were also rejected under 35 U.S.C. §112, First Paragraph, on the grounds that the specification for lack of enablement. Specifically, the Office Action states that

> Apart form those embodiments specifically disclosed in the specification for use in up-regulating antitumor agents in tumor cells, while down-regulating those same agents in normal cells through a p53 pathway (i.e. using either HSP70-, MDR1-, PCNA promoters or variants thereof; or possibly $p16^{INK4A}$ pathway), the specification fails to provide sufficient guidance teaching how to make or use any other compositions for use in a non-p53 based method or in accordance with the composition of claim 1...(see page 6, first paragraph of the Office Action).

Applicants submit that this rejection has been obviated in view of the amendment to claim 1. As discussed above, claim 1 has now been amended to specify, in pertinent part, "...a first promoter whose function is suppressed *by wild-type p53 or* $p16^{INK4}$ in non-tumor cells relative to tumor cells *carrying a mutant p53 or p16 allele*..." Therefore, reconsideration and withdrawal of the rejection of claims 1-24 is respectfully requested.

Claims 20-23 have been further rejected for "embracing embodiments that read on *in vivo* gene therapy which is not enabled by the instant application." Specifically, the Office Action states that

> When read in light of the specification, the only substantial or well-established utility that can be gleaned from the claimed compositions or methods of claims 20-23 is gene therapy for cancer. Absent any evidence to any other non-therapeutic utility, for purposes of enablement, claims 20-23 were evaluated to the extent that the specification provides an enabling disclosure for *in vivo* gene therapy (see page 8, first paragraph of the Office Action).

The Office Action further states that

> Absent evidence of reduction to practice in the specification or declaratory post-filing evidence of such using the methods and guidance disclosed in the instant application, there is no expectation of success for the claimed subject matter as it related to gene therapy (see page 8, second paragraph of the Office Action).

Applicants respectfully traverse this rejection. First, the utility of the claimed invention is not limited to gene therapy. In fact, the specification is replete with examples of non-therapeutic utilities for the present invention. For example, numerous examples are provided for using the compositions and methods of the present invention in *in vitro* applications, such as determining the transcriptional activity of normal and mutant p53 alleles in cell lines such as K562 and SAOS-2 cells, monitoring the p53-dependent killing by pro-drug activation and assessing cytotoxicity through cell-killing assays, such as TK-GCV cell killing assays (see, *e.g.*, page 31, line 20 through page 61, line 20; Tables 1 – 6 at pages 62-67; and Figures 6-10). All of these assays are substantial and well-established utilities that are enabled by the present specification.

Moreover, with regard to the use of the present invention in gene therapy, as disclosed in the specification, Applicants submit herewith Lipinski *et al.* (Lipinski *et al.* (2001) *Gene Therapy* 8:274-281; attached hereto as Appendix C) as post-filing evidence of the reduction to practice of the methods of the present invention. Lipinski *et al.* describe the *in vivo* use of nucleic acid constructs disclosed in the present invention, namely a viral vector that contains a dual promoter

system comprising a first promoter whose function is suppressed in non-tumor cells relative to tumor cells and second promoter which is upregulated in non-tumor cells. Specifically, the viral vector utilized by Lipinski *et al.* contains two independent expression cassettes: (1) the *E. coli* nitrotransducase gene (a pro-drug converting enzyme) driven by the human HSP70 promoter containing the LacI binding site and (2) a p53-inducible lac repressor gene. Applicants respectfully submit that the Type I construct used in Lipinski *et al.* includes the HSP70 promoter in combination with lacO sequences and the Type II construct includes the $tkGC_3$ promoter, as disclosed in the present specification (see, *e.g.*, Figure 2 of Appendix C and page 35 of the specification). Lipinski *et al.* demonstrate that co-administration of the viral vector with systemic pro-drug significantly reduces tumor size and survival compared with the control (see, *e.g.*, paragraph bridging pages 276-277 of Appendix C). Moreover, a high level of repression of the pro-drug activating enzyme was demonstrated for non-tumor cells even when the viral vector was administered via intravenous injection. Finally, Lipinski *et al.* also disclose that the performance of the viral vector is due, in part, to the ability of the adenovirus infection to upregulate wildtype p53 in non-tumor cells (see, *e.g.*, the paragraph bridging pages 277 and 278 of Appendix C). This enhancement of discrimination is disclosed in the instant specification, which states "discrimination between normal and tumor cells may be enhanced by the introduction of wildtype p53 which takes place in normal cells upon infection with a DNA tumor virus" (see, *e.g.*, page 61, lines 12-15 of the specification).

Therefore, Lipinski *et al.* demonstrate that the present invention can be performed *in vivo* by the routine techniques of intra-tumoral or intravenous administration, based on the teachings disclosed in the instant specification. The fact that a different pro-drug is used in Lipinski *et al.* is irrelevant. Furthermore, Lipinski *et al.* also demonstrate that the properties of the composition of the present invention *per se* are sufficient to overcome the difficulties of targeting, delivery and selectivity as stated in the Office Action.

Thus, it is Applicants position that in view of the Lipinski *et al.* reference and the teachings disclosed in the specification and claims of the present invention, it would require no more than routine experimentation by one of ordinary skill in the art to make and/or use the claimed invention. Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

CONCLUSION

On the basis of the foregoing amendments and remarks, Applicants respectfully submit that the pending claims are in condition for allowance. If a telephone conversation with Applicants' Attorney would expedite prosecution of the above-identified application, the Examiner is invited to call the undersigned at (617) 227-7400.

Respectfully submitted,

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APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

1. (Amended) A composition comprising a first nucleic acid construct comprising a first gene whose expression is controlled by a first promoter whose function is suppressed by a wild-type p53 or $p16^{INK4}$ allele in non-tumor cells relative to tumor cells carrying a mutant p53 or p16 allele, and a second nucleic acid construct comprising a second gene whose gene product suppresses expression of said first gene, wherein the expression of said second gene is controlled by a second promoter that is up-regulated in non-tumor cells relative to tumor cells carrying a mutant p53 or p16 allele, such that said first gene is expressed in tumor cells and suppressed in non-tumor cells.

15. The composition according to claim ~~14~~ 1 wherein said first promoter is the HSP70 promoter.

APPENDIX B
PENDING CLAIMS

1. A composition comprising a first nucleic acid construct comprising a first gene whose expression is controlled by a first promoter whose function is suppressed by a wild-type p53 or $p16^{INK4}$ allele in non-tumor cells relative to tumor cells carrying a mutant p53 or p16 allele, and a second nucleic acid construct comprising a second gene whose gene product suppresses expression of said first gene, wherein the expression of said second gene is controlled by a second promoter that is up-regulated in non-tumor cells relative to tumor cells carrying a mutant p53 or p16 allele, such that said first gene is expressed in tumor cells and suppressed in non-tumor cells.

2. The composition according to claim 1 wherein said second gene of said second nucleic acid construct encodes an antisense RNA transcript complementary to a sequence within mRNA encoded by said first gene of said first nucleic acid construct.

3. The composition according to claim 1 wherein said second gene of said second nucleic acid construct encodes a ribozyme specific for a sequence within mRNA encoded by said first gene of said first nucleic acid construct.

4. The composition according to claim 1 wherein said second gene of said second nucleic acid construct encodes a sequence-specific transcriptional suppressor and said first nucleic acid construct comprises a binding site recognized by said sequence-specific transcriptional suppressor.

5. The composition according to claim 4 wherein said sequence-specific transcriptional suppressor is a *lac* operator suppressor.

6. The composition according to claim 4 wherein said sequence-specific transcriptional suppressor comprises a *tet* repressor DNA-binding domain and a transcriptional suppression domain of the *Drosphilia* KRAB transcription factor.

7. The composition according to claim 4 wherein said sequence-specific transcriptional suppressor comprises a Gal-4 DNA-binding domain and a transcriptional suppression domain of the *Drosphilia even-skipped* transcription factor.

8. The composition according to claim 1 wherein said first nucleic acid construct and said second nucleic acid construct are each on separate nucleic acid vectors.

9. The composition according to claim 1 wherein said first nucleic acid construct and said second nucleic acid construct are on a single nucleic acid vector.

10. The composition according to claim 9 comprising an insulator sequence between said first nucleic acid construct and said second nucleic acid construct.

11. The composition according to claim 10 wherein said nucleic acid vector is a viral vector.

12. The composition according to claim 1 wherein said second promoter of said second nucleic acid construct comprises a p53 binding site sequence or CMV promoter.

13. The composition according to claim 12 wherein said second nucleic acid construct comprises said p53 binding site sequence downstream of a TATA Box and downstream of the transcriptional start site of said second promoter of said second nucleic acid construct.

15. The composition according to claim 1 wherein said first promoter is the HSP70 promoter.

16. The composition according to claim 1 wherein said first gene is a reporter gene.

17. The composition according to claim 1 wherein said first gene encodes an antitumour agent.

18. The composition according to claim 17 wherein said antitumour agent is a pro-drug activating enzyme.

19. The composition according to claim 18 wherein said pro-drug activating enzyme is a thymidine kinase.

20. A cell containing a first nucleic acid construct and a second nucleic acid construct of a composition according to claim 1.

21. The cell according to claim 20 which is a tumor cell.

22. A method comprising introduction of a first nucleic acid construct and a second nucleic acid construct of a composition according to claim 1 into a cell.

23. The method according to claim 22 wherein said cell is a tumor cell.

24. The method according to claim 23 wherein said first nucleic acid construct and said second nucleic acid construct are introduced into said cell *in vitro*.

25. The composition of claim 1, wherein said first promoter is selected from the group consisting of the HSP70 promoter, the Bcl-2 promoter, the PCNA promoter, the MDR1 promoter, the CMV promoter and the $p16^{INK4}$ promoter.

Gene Therapy (2001) 8, 274–281

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RESEARCH ARTICLE

Tumour-specific therapeutic adenovirus vectors: repression of transgene expression in healthy cells by endogenous p53

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Approximately 50% of human tumours lack functional p53 suppressor protein. A promoter that is repressed by p53 in healthy cells could thus provide tumour-specific gene expression for a huge subset of tumours. In this report we describe a double recombinant adenovirus vector, 'Ad.p53R', encoding a therapeutic gene that is indirectly repressed by endogenous wild-type p53. Ad.p53R contains two independent expression cassettes; (1) the E. coli nitroreductase gene (NTR) driven by the human hsp70 promoter containing LacI binding sites (hsp70lacO-NTR) and (2) a p53-inducible lac repressor gene (tkGC$_3$-lacI). In p53 null cells (Hep3B), Ad.p53R directed the same level of NTR expression as Ad.p53NR which lacks the tkGC$_3$-lacI cassette. Moreover, injection of SW480 xenografts (mutated p53) with Ad.p53R resulted in a clear inhibition of growth in response to the prodrug CB1954. In cells retaining wt p53 (HepG2 and primary human endothelial cells), Ad.p53R expressed significantly less NTR (approximately 70%) than Ad.p53NR. Ad.p53R administered by i.v. injection also produced significantly less NTR than Ad.p53NR in normal tissues in vivo. Finally, adenovirus infection per se of cultured HepG2 cells at low MOI induced p53 stabilisation suggesting that adenovirus-mediated gene delivery may contribute to p53-based selectivity. Gene Therapy (2001) **8**, 274–281.

Keywords: *adenovirus; transcriptional repression; p53; Lac operon*

Introduction

Adenoviral vectors provide the most efficient gene transfer to tumour tissue *in vivo* that is currently achievable and are thus the lead vectors for cancer gene therapy clinical trials. On the other hand, because of their very broad tissue tropism, they have the potential to deliver therapeutic genes to, and damage, healthy tissue. Tumour-specific gene therapy can theoretically be achieved by changing the natural adenoviral tropism by genetic modification of the adenovirus fibre[1–3] by using antibody-ligand complexes[4,5] or bi-specific antibodies[6] and/or by using tumour-specific promoters.[7] Fibre modification can however result in lower virus yield[8] and tumour-specific promoters are at the moment available only for some tumour types and are often not sufficiently active to drive therapeutic levels of transgene expression.[9] To better exploit the positive features of adenovirus vectors for cancer therapy there is a need for a more generic means of obtaining therapeutic levels of cancer cell-specific transgene expression that is compatible with well-established and highly productive adenovirus production techniques.

The tumour suppressor gene p53 is mutated in or absent from about 40–80% of human tumours depending on the origin of the tumour.[10] Loss of p53 function is thought to result in profound alterations in gene expression including both increased and decreased expression of target genes involved in cellular homeostasis (DNA replication and repair, cell cycle progression and apoptosis induction). Recently a novel gene control system based on the ability of p53 to regulate target gene expression has been described.[11] The key feature of this system is that it harnesses p53 present in healthy cells to actively repress therapeutic gene expression. P53-mediated repression of a luciferase gene carried on a plasmid was achieved by cotransfection with a second plasmid carrying a p53-inducible gene encoding a repressor of the promoter driving luciferase expression. The authors exemplify the use of different repressor systems including p53-inducible LacI expression combined with an *hsp70* promoter fused to three downstream LacI binding sites. Exploitation of this system for cancer gene therapy will, however, require that the therapeutic and repressor gene cassettes be combined into an efficient gene delivery vector within which their distinct modes of regulation are preserved, in particular the dependence of the repressor-encoding cassette on wild-type p53. In this report we describe a double recombinant adenovirus based on the above system that expresses the *E. coli* NTR prodrug-converting enzyme selectively in cancer cells lacking p53 function.

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Received 9 June 2000; accepted 8 December 2000

Results

Activation of a synthetic p53-responsive promoter ('tkGC$_3$') by endogenous p53

A previous study describes a synthetic promoter, 'tkGC$_3$', that is induced by transiently expressed p53 protein.[12] Before beginning recombinant adenovirus construction we determined the level of inducibility of this promoter by endogenous p53 protein and its basal activity in cells lacking functional p53. Human cell lines containing wild-type p53 (HepG2 hepatocellular carcinoma), mutant p53 (SW480 colorectal carcinoma (G/A273, C/T309) and DU-145 prostate carcinoma (P/L274, V/F223)) or lacking p53 protein entirely (Hep3B hepatocellular carcinoma) were used for this. The point mutations at nucleotide positions 223, 273 and 274 are located within the p53 DNA-binding domain and normally prevent DNA binding. The cell lines were transiently transfected with a ptkGC$_3$-luc or CMV-luc reporter plasmid and luciferase expression levels compared. In Figure 1 we show that the tkGC$_3$ promoter was as active as the CMV promoter/enhancer in HepG2 cells and significantly less active than CMV in cell lines lacking functional p53.

Adenovirus-mediated gene expression: inverse correlation with p53 status

Having established that the tkGC$_3$ promoter is highly inducible by endogenous p53 as well as by the transiently expressed protein,[12] we constructed a double recombinant Ad5, 'Ad.p53R' (p53-repressible gene expression) containing a tkGC$_3$-lacI expression cassette in the deleted E3 region and the *E. coli* nitroreductase gene (NTR) driven by the *hsp70* promoter in the deleted E1 region (Figure 2). The latter contained LacI-binding sequences within an intron located between the promoter and the NTR open reading frame. We also constructed two control viruses lacking the tkGC$_3$-lacI expression cassette. 'Ad.p53NR' (p53 non-repressible) contains the NTR gene driven by the *hsp70* promoter with downstream LacI binding sites. 'Ad.p53NR(-lacO)' contains the NTR gene driven by an unmodified *hsp70* promoter. In Figure 3 we show that the tkGC$_3$ promoter retained its specificity and p53-inducibility within the adenovirus vector. Ad.p53R-infected HepG2 cells (wt p53) expressed approximately 200-fold more LacI protein than Ad.p53R-infected Hep3B cells (p53 null) which, in turn, expressed a level close to background. P53-induced LacI expression in Ad.p53R-infected cells containing wt p53 resulted in a significant down-regulation (approximately 70%) of the hsp70lacO-NTR cassette located in the deleted E1 region of the virus. In Figure 4a we show that HepG2 cells (wt p53) infected with Ad.p53R (hatched bar) expressed significantly less NTR protein than control cells infected with Ad.p53NR, which lacks the cassette but is otherwise identical (grey bar). In contrast, infection of p53-null Hep3B with these viruses resulted in very similar levels of NTR expression. These ELISA data were independently validated by Western blot analysis. Densitometric scanning indicated an 80% lower level of NTR expression in HepG2 cells infected with Ad.p53R compared with control cells

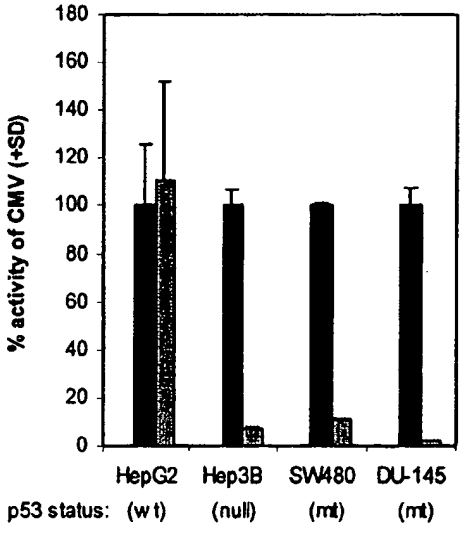


Figure 1 Induction of the tkGC$_3$ promoter by endogenous p53. Luciferase activity was measured in whole cell extracts of HepG2, Hep3B, SW480 and DU-145 cells prepared 48 h after transfection with 1 μg of pGL3basic, CMV-Luc (black bars) or ptkGC$_3$-Luc (grey bars). Activity is expressed as percentage of CMV-Luc activity in the respective cell line. The error bars denote the standard error of the mean value of double determinations (two independent experiments).

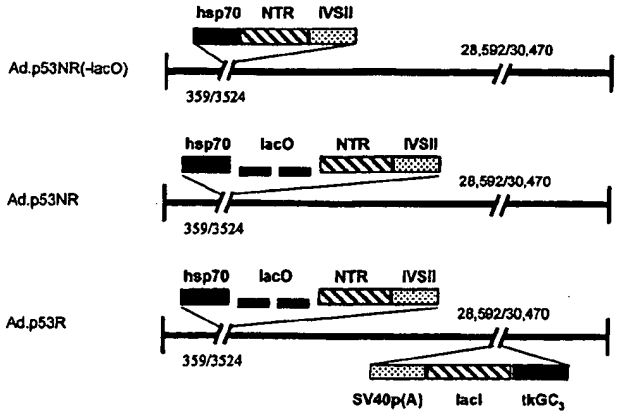


Figure 2 Structures of E1 and E1/E3 recombinant adenoviruses. The numbers indicate the locations of the E1 and E3 deletions. Hsp70, human heat shock protein promoter nt −117 to +26; NTR, E. coli nitroreductase gene; IVSII, human β-globin intron II; lacO, intron containing two LacI binding sites; tkGC$_3$, promoter consisting of the upstream basal tk promoter (nt −109 to +52) combined with the GC$_3$ binding element GCCC(GGACTTGCCT)$_3$; LacI, bacterial lac repressor gene; SV40p(A), SV40 late gene polyadenylation signal.

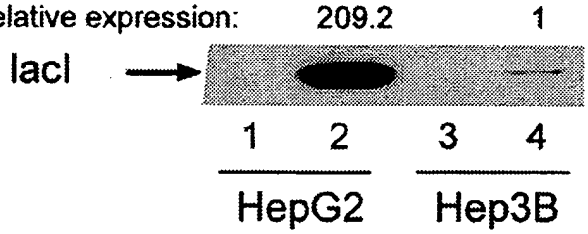


Figure 3 Western blot analysis of nuclear LacI protein in Ad.p53R-infected cells. Nuclear extracts were prepared from HepG2 and Hep3B cells 48 h after infection (MOI = 20 p.f.u. per cell) with Ad.null (lanes 1 and 3) or with Ad.p53R (lanes 2 and 4). 30 μg of each extract was separated and probed for LacI protein by Western blotting as described in Materials and methods.

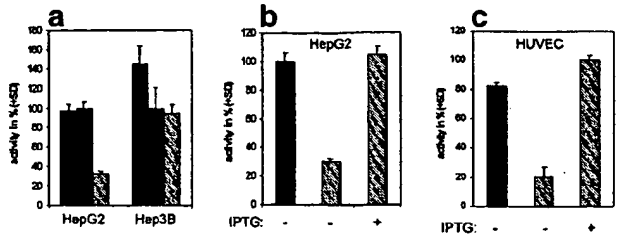


Figure 4 LacI-mediated repression of NTR expression by p53 in Ad.p53R-infected HepG2 cells and primary human endothelial cells (a) HepG2 and Hep3B cells were infected with Ad.p53NR(-lacO) (black bars), Ad.p53NR (grey bars) or Ad.p53R (hatched bars) at an MOI of 50 p.f.u. per cell and NTR expression determined by ELISA as described in Materials and methods. Data are from three independent experiments. Error bars show standard deviation (s.d.). (b) HepG2 cells were infected with Ad.p53R in the presence of IPTG (+) and NTR expression determined by ELISA as described in Materials and methods. Control cells were infected with either Ad.p53NR or with Ad.p53R without IPTG treatment. NTR expression levels are expressed as a percentage of mean Ad.p53NR-directed NTR expression. Data are from two independent experiments. Error bars show standard deviation. (c) HUVEC were infected and analysed as in (b). Data are from two independent experiments.

infected with Ad.p53NR (data not shown). Direct evidence for LacI-mediated repression of the hsp70lacO-NTR expression cassette was provided by inclusion of IPTG in the culture medium of HepG2 cells infected with Ad.p53R to prevent LacI:*lacO* interaction. Cells infected with the double recombinant virus in the presence of IPTG expressed NTR at the same level as those infected with the Ad.p53NR control virus (Figure 4b).

Surprisingly, a comparison of expression levels obtained with Ad.p53NR *versus* Ad.p53NR(-lacO) in HepG2 and Hep3B cells showed that addition of LacI-binding sequences to the *hsp70* promoter had no effect on its activity in HepG2 cells, whereas the modified promoter was approximately 30% less active in Hep3B cells (Figure 4a). We did not investigate this effect further.

As well as using a tumour cell line that retains wt p53 to demonstrate repression of therapeutic gene expression by p53 we detected an equivalent repression by endogenous p53 in cultured primary human umbilical vein endothelial cells (HUVEC). NTR expression was significantly lower in Ad.p53R-infected cells compared with Ad.p53NR-infected cells. As shown using HepG2 cells this difference in expression level was eliminated when the infection with Ad.p53R was done in the presence of IPTG to block the action of p53-induced LacI protein (Figure 4c).

Anti-tumour effects of Ad.p53R in a xenograft model of human colon cancer

Expression of NTR in cancer cell lines *in vitro* results in cell death in the presence of the prodrug CB1954 which is converted by NTR into a potent DNA cross-linking agent.[13,14] Intratumoral (i.t.) injection of an adenovirus vector containing a CMV.NTR expression cassette resulted in a significant anti-tumour response to systemically administered (intraperitoneal) CB1954 for a range of solid tumour models.[15] In Figure 5 we show that i.t. injection of Ad.p53R combined with systemic CB1954 administration resulted in clear anti-tumour effects (retardation of tumour growth and extended host mouse survival) in the SW480 xenograft model of colorectal cancer (p53 mutated). Whereas by 28 days after commencement of the study mean control tumour size increased on average by 85%, tumours that received both Ad.p53R and CB1954 were on average only 22% larger (Figure 5a). Ad.p53R injection and CB1954 treatment also resulted in

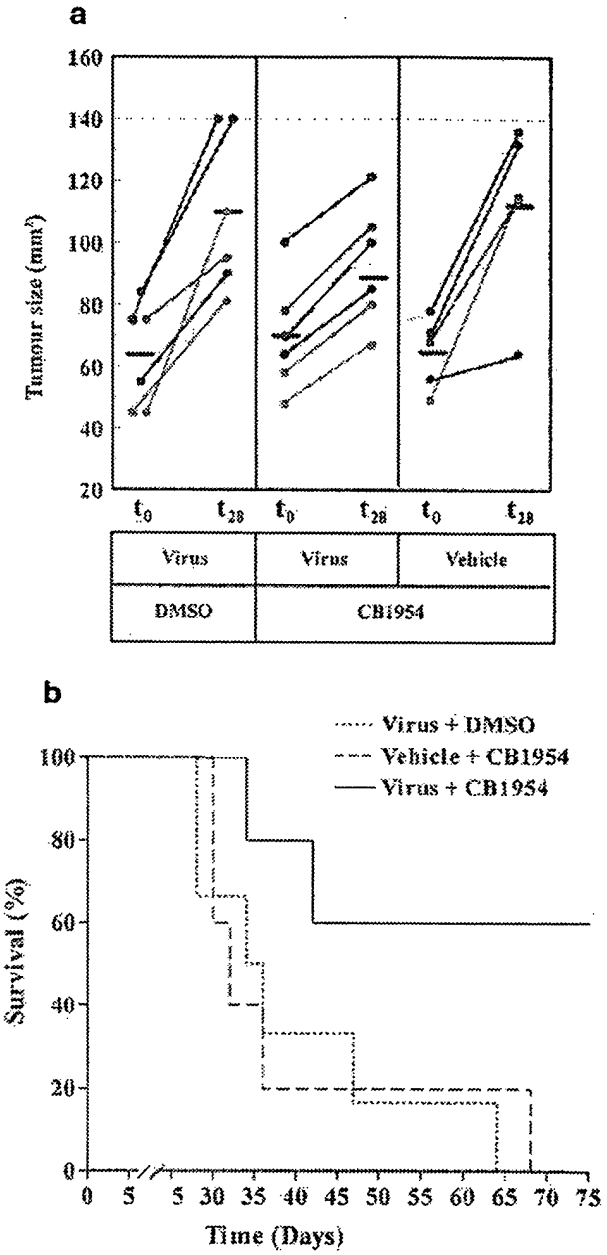


Figure 5 Anti-tumour efficacy of Ad.p53R in a xenograft model of colon cancer. (a) Symbols represent individual SW480 tumour sizes and horizontal bars represent the mean tumour sizes for each experimental group before virus injection and 28 days after injection. Tumours were injected with 3.2×10^{10} particles of Ad.p53R and CB1954 treatment started 48 h later and continued for a total of 5 consecutive days. Control animals received either virus and prodrug vehicle or virus vehicle and CB1954. (b) Kaplan–Meier survival plot showing percentage surviving animals (tumours less than 140 mm²) versus time. Day 0 corresponds to the first day of administration of CB1954.

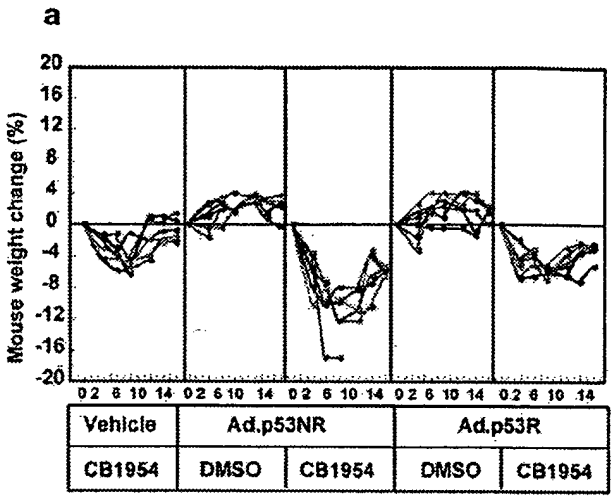
a significant prolongation of survival of tumour-bearing mice compared with control treatment (Figure 5b).

Down-regulation of NTR expression in vivo

The preceding data demonstrate that Ad.p53R can provide therapeutic benefit in a model lacking p53 function and, based on the use of cultured cells, that p53-induced LacI expression can significantly down-regulate NTR expression in cells containing functional p53. In Figure 6 we provide strong evidence that this p53-mediated gene repression can effectively prevent NTR expression in normal cells *in vivo*, in particular the liver, the tissue most at risk from adenovirus disseminated from an injected tumour.[16] To mimic accidental virus dissemination, Ad.p53R or Ad.p53NR control virus (10^8 particles) were administered to groups of normal mice ($n = 5$) by tail vein injection. After a 48 h period to allow gene expression in infected tissues, CB1954 or prodrug vehicle were administered to the mice by i.p. injection. A further group of mice ($n = 5$) received only virus vehicle followed by CB1954. Body weight loss for each mouse was monitored as a surrogate measure of systemic toxicity caused by prodrug activation in infected tissues and plotted as a function of time. As shown in Figure 6a, injection of Ad.p53NR control virus and CB1954 resulted in an apparently significant increase in toxicity compared with mice that received only prodrug. Comparison of the mean maximum weight loss for the two groups (dead mouse excluded) using the Student's *t* test confirmed this ($P < 0.02$). In contrast, injection of Ad.p53R resulted in a pattern of body weight loss very similar to that caused by prodrug alone except that weight recovery occurred more slowly. Student's *t* test analysis of mean maximum weight loss confirmed no significant difference between these groups ($P > 0.07$). To obtain more direct evidence for down-regulation of NTR expression in normal tissue *in vivo*, the livers of additional mice were excised 48 h after tail vein injection with either Ad.p53R or Ad.p53NR (1×10^{11} virus particles), sectioned and NTR expression determined by NTR-immunostaining.[17] Representative data are shown in Figure 6b. In agreement with the toxicity data described above, whereas infection with Ad.p53NR resulted in clearly detectable NTR expression in the liver, infection with Ad.p53R generated an NTR expression level just above background using indirect immunoperoxidase staining.

P53 induction by E1-deleted adenovirus infection may contribute to the down-regulation of NTR expression in healthy cells

It is well documented that expression of E1 proteins following the infection of cells with wild-type adenovirus results in accumulation of p53.[18] Infection of cells with E1-deleted replication defective adenoviral vectors can also induce p53.[19] This property of adenovirus vectors has the potential to enhance the degree of selectivity of the Ad.p53R virus for cancer cells that lack functional p53 by increasing the level of p53 in healthy cells and consequently the level of LacI. We determined the level of p53 in nuclear extracts of untreated HepG2 cells infected with increasing doses of Ad.null vector using Western blotting. In Figure 7 we show that infection of HepG2 cells with an MOI of only 1 p.f.u. per cell resulted in a clear increase in the level of nuclear p53 above the very low basal level detected in control cells. With higher doses of



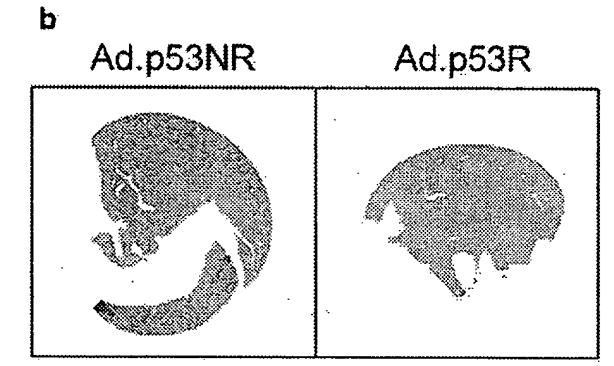


Figure 6 In vivo down-regulation of NTR expression by p53-induced LacI (a) Nude mice were i.v. injected with either Ad.p53NR or with Ad.p53R (1×10^8 particles) and subsequently treated with either CB1954 or prodrug vehicle as described in Materials and methods. A fifth group was injected with virus vehicle and with prodrug. The graph shows individual mouse body weight plotted as a function of time. Control mice received virus vehicle before CB1954 administration. Group size was five for each treatment regimen. (b) Nude mice ($n = 4$) were i.v. injected with either Ad.p53NR or with Ad.p53R (1×10^{11} particles), killed after 48 h and livers removed for NTR immunostaining as previously described.[17]

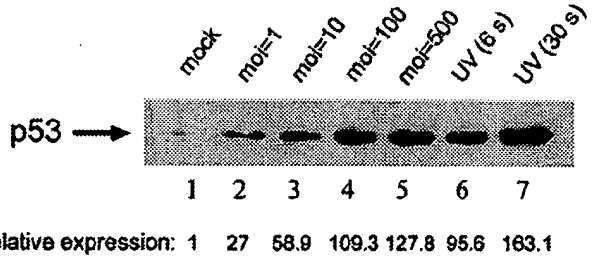


Figure 7 Induction of nuclear p53 protein by infection with an E1/E3-deleted adenovirus at low MOI. Nuclear extracts were prepared from HepG2 cells 48 h after infection with Ad.null (MOI = 0–500 p.f.u. per cell) and analysed for nuclear p53 protein (35 μg) by Western blotting as described in Materials and methods. For UV induction of p53, cells were irradiated 24 h after mock-infection (6 or 30 s at a dose of 2500 J/cm²) and harvested 24 h later.

virus up to 500 p.f.u.per cell the level of p53 was further increased to a level comparable to that induced by UV irradiation.

Discussion

The development of recombinant adenoviruses that express therapeutic levels of anti-cancer genes in a wide range of tumour types but at low levels in healthy cells would significantly increase the utility of adenovirus-mediated cancer gene therapy. A recent report[11] describes transient transfection experiments exemplifying a novel p53-mediated gene regulation system that could potentially be used in about 50% of human cancers that lack functional p53.[10] The basis for this system is that the majority of cancer cells with mutated p53 and all p53-null cancer cells will be unable to activate a p53-dependent repressor gene cassette allowing a therapeutic gene cassette that is a target for the repressor to express at maximum level in these diseased cells. The p53 protein present in infected healthy cells, however, will induce repressor synthesis resulting in the blockade or at least significant attenuation of therapeutic gene expression. In this report we demonstrate that the system works within a recombinant adenovirus vector expressing *E. coli* nitroreductase which activates the prodrug CB1954. Antitumour effects were observed in a xenograft model of colorectal cancer lacking functional p53 and endogenous p53 was shown to provide therapeutic gene repression in cell culture models and, apparently, also in healthy tissue *in vivo*.

In transient transfections, the $tkGC_3$ promoter used in our study was shown to be highly inducible by endogenous p53 (induced level comparable to CMV). This could not necessarily have been predicted from previous studies with this promoter as these used transiently expressed p53 (International Patent Application WO 97/12970). Crucially, the $tkGC_3$ promoter remained p53-dependent and highly inducible by p53 when cloned into the E3 region of Ad.p53NR to create the double recombinant virus, Ad.p53R. This retention of p53-dependence indicates that the 25 kb of adenovirus sequences separating the $tkGC_3$-lacI and hsp70lacO-NTR cassettes provided effective insulation with no apparent influence of adenoviral promoter elements. In fact the very low basal LacI expression level in Hep3B cells and high expression level in HepG2 cells suggest that the selectivity of the promoter was increased in the context of the adenovirus E3 region. P53-dependent LacI expression was able to attenuate NTR expression significantly in cells expressing functional p53. Hep3B cells (p53 null) infected with Ad.p53R expressed the same level of NTR protein as cells infected with Ad.p53NR which lacks the repressor cassette whereas Ad.p53R-infected HepG2 cells and primary HUVEC (p53 wild-type) expressed significantly less NTR protein than control cells infected with Ad.p53NR.

Significantly, infection of cultured cells with replication-defective adenovirus was found to induce p53 accumulation even at low MOI. It is thus likely that adenovirus-mediated gene delivery will enhance the ability of the present system to discriminate between cancer and non-cancer cells. P53-induction by low levels of adenovirus would be valuable when the therapeutic gene encodes a highly potent product. The results of *in vivo* infection with Ad.p53R and Ad.p53NR provide strong evidence that p53-mediated gene repression can provide selectivity *in vivo* as well as in cultured cells. Infection with Ad.p53R followed by CB1954 administration resulted in significantly less toxicity than that observed following the injection of Ad.p53NR. Consistent with this, NTR expression was significantly higher in the livers of mice injected with Ad.p53NR.

The decision to use the human *hsp70* promoter to drive transcription was based on previous reports that it is a target for direct regulation by p53 protein, either repression by wt p53 in normal cells or activation by many of the mutated p53 forms found in humans.[20] Other promoters sharing these properties include the *CMV, PCNA, RSV, HTLV-I LTR, bcl-2* and *MDR-1* promoters.[12] An additional potential benefit of the *hsp70* promoter is that it can be activated by conditions associated with cancer such as hypoxic stress,[21] as well as by therapeutic induction of hyperthermia. Importantly, incorporation of the LacI-binding sites downstream of the *hsp70* promoter fragment used in the study rendered it repressible by LacI protein expressed from the $tkGC_3$-lacI cassette in HepG2 cells. An alternative explanation that the promoter was simply less active in the context of the double recombinant virus was clearly ruled out by inclusion of IPTG in the cell culture medium which restored its activity to the same level observed in the absence of the $tkGC_3$-lacI cassette. Interestingly, whereas the *hsp70*lacO promoter was as active as the unmodified *hsp70* promoter in HepG2 cells, it was consistently less active than the native promoter fragment in the p53 null Hep3B line. We currently have no explanation for this apparent cell type-specific effect of inserting LacI-binding sites downstream of the *hsp70* promoter.

The NTR ELISA data from HepG2 cells and HUVEC infected with Ad.p53R show that even a high level of LacI did not result in total repression of the NTR gene. A more complete repression may be achieved by increasing the number of *lac* operator sequences, by use of a higher affinity LacI mutant such as Lac^{his},[22] or by a combination of these two modifications. Alternatively the location of the LacI binding sites with respect to the *hsp70* promoter may be a critical factor and repression by LacI may be improved by optimising this. Another likely explanation for the residual level of NTR expression is that this was synthesised before the expression of inhibitory levels of LacI protein. The amount of pre-repression synthesised protein could be reduced by insertion of a sequence such as PEST[23] to reduce the half-life of the protein. A more generic modification would be to substitute the *hsp70* promoter with an inducible promoter, for instance one induced by tetracycline.[24] Following administration of the virus, after a period for LacI expression the recipient would begin treatment with tetracycline to activate therapeutic gene expression in cancer cells lacking functional p53. On the other hand, a very effective repression of NTR expression was observed *in vivo*. Further experimentation may show that the present system provides an adequate level of selectivity, at least for NTR expression. A problem with the use of the *lac* repressor system in a clinical setting is that the protective effect of LacI expression in healthy tissue is likely to be offset by the generation of cytotoxic T lymphocytes against infected healthy cells. A ribozyme[25] that specifically degrades the therapeutic gene transcript is the most obvious candidate for use in a clinical setting. As well as

escaping immune detection, a suitably optimised ribozyme should provide more effective repression because of its catalytic mode of action. It would also be synthesised more rapidly than LacI, resulting in an earlier establishment of repression. To maximise the utility of such a ribozyme this should be targeted to a region outside the therapeutic gene, for instance to the 5′ or 3′ untranslated regions.

In conclusion, we have demonstrated that a p53-regulated transgene expression system involving independently functioning therapeutic and p53-inducible expression cassettes can be successfully incorporated into a recombinant adenovirus vector. Realisation of the full potential of this system is likely to require the use of a non-immunogenic repressor molecule. The system is also likely to be most effective for therapeutic molecules with short half-lives or for those whose half-life can be reduced by appropriate manipulation. A further motivation to further develop this system is that as well as providing generic cancer cell-specific gene expression it also offers the advantage that it is unlikely to select escape mutants (ie tumour cells would be unlikely to regain wt p53 function).

Materials and methods

Cell culture

The cell lines HepG2, Hep3B, SW480 and DU-145 were obtained from ATCC (Manassas, VA, USA). PER.C6 cells[26] were obtained from IntroGene (Leiden, The Netherlands). HUVEC were supplied by Promocell (Heidelberg, Germany). All lines were maintained as recommended by the supplier.

Plasmid construction

ptkGC$_3$-luc was constructed (1) by cloning the *tk* promoter from pT-109[27] as a *ScaI*/*BglII* fragment into *SmaI*/*BamHI* digested pCR-script (Cam)SK(+) (Stratagene, La Jolla, CA, USA) to create ptk-SK; (2) by cloning the GC$_3$ element (GCCC(GGACTTGCCT))$_2$ from the human Rb gene promoter[28] into the unique *EcoRV* site of ptk-SK to create ptkGC$_3$-SK; and (3) by transferring the tkGC$_3$ promoter element as a *SacI*/*HindIII* fragment into *SacI*/*HindIII*-cut pGL3basic (Promega, Madison, WI, USA). CMV-luc was constructed by cloning the human CMV-IE promoter fused to luc+ into *SmaI* cut pSW107. pSW107 was constructed by cloning a 917 bp fragment of the human β-globin gene (*BamHI* site in exon 2 to the *EcoRI* site in exon 3) coupled to a 240 bp *HincII*–*BamHI* fragment containing the poly(A) addition and transcriptional termination signals of the human complement C2 gene into pBluescript (Stratagene).

The hsp70-NTR expression cassette was constructed by PCR amplification of the human *hsp70* promoter (nt −117 to +26) using hsp70-lacO$_3$-rL[12] as template and insertion as a *HindIII* fragment into *HindIII*-cut pTX0374.[15] The cassette was then cloned into the adenovirus transfer vector pPS1128 which contains adenoviral sequences from the left hand ITR to nt 359 and from nt 3525 to 10 589 to create pPS1128.hsp70-NTR. pPS1128.hsp70lacO-NTR was constructed (1) by cloning a *HindIII* fragment containing two LacI binding sites within an intron from pOPI3CAT (Stratagene) into *HindIII*-digested pTX0374; (2) by cloning the PCR-amplified hsp70 promoter (nt −117 to +26) as a *ClaI* fragment upstream of the LacI binding sites and (3) by cloning the complete expression cassette as a *SpeI* fragment into pPS1128. Both pPS1128/hsp70-NTR and pPS1128/hsp70lacO-NTR contain the expression cassette in a left-to-right orientation.

pPS1160.ptkGC$_3$-lacI was constructed (1) by cloning a blunted *XhoI*/*SalI* fragment from ptkGC$_3$-lacI into *SalI*/blunted pCR-Blunt/Linker resulting in pCR-Blunt/tkGC$_3$-lacI. The tkGC$_3$-lacI expression cassette was then cloned as a *PacI* fragment into *PacI* prepared pPS1160[15] to create pPS1160.tkGC$_3$-lacI which contains the *lacI* cassette in a right-to-left orientation. pCR-Blunt.Linker was constructed by insertion of an additional *PacI* cloning site into pCR-Blunt (Invitrogen, Groeningen, The Netherlands). The 1667 bp *XbaI*/*HindIII* fragment of p3′SS (Stratagene) containing the *lacI* gene fused to a nuclear localisation signal was cloned at the *EcoRV* and *HindIII* sites of the pCR-script SK(+)(Cam) (Stratagene) to create pCR-lacI. The 1.7 kb *EcoRI*/*SalI* fragment of pCR-lacI was subcloned into the *HindIII*/*SalI* sites of ptkGC$_3$-SK to create ptkGC$_3$-lacI.

Construction and growth of recombinant adenoviral vectors

Ad.p53NR(-lacO) and Ad.p53NR were constructed by cotransfection of PER.C6 cells at 90% confluence with an equimolar mixture of pPS1160 and respectively, pPS1128.hsp70-NTR or pPS1128.hsp70lacO-NTR. Ad.p53R was constructed by cotransfection with a mixture of pPS1128.hsp70lacO-NTR and pPS1160.tkGC$_3$-lacI. All plasmids were *SwaI*-linearised before transfection. Recombinant viruses were harvested about 7–10 days later at maximum CPE by three freeze–thaw cycles in infection medium (DMEM, 1% FCS, 2 mM $MgCl_2$). The viruses were then expanded and purified by standard CsCl density centrifugation. Banded virus was dialysed against an excess of storage buffer (10 mM Tris pH 7.4, 140 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5% sucrose), snap-frozen in aliquots in liquid nitrogen and stored at −80°C. Particle concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Plaque-forming units (p.f.u.)/ml were determined by standard plaque assay.

Transfections and luciferase assays

HepG2, Hep3B, SW480 and DU-145 were seeded the day before transfection at 2.5×10^5, 1.0×10^5, 1.5×10^5 and 2.0×10^5 cells per six-well, respectively. The next day, a 2:1 ratio (μg:μg) of peptide CL22[29] and plasmid DNA was mixed together in a final volume of 100 μl in HBS (10 mM Hepes pH 7.4, 150 mM NaCl; Sigma, Willingham, UK) and incubated at RT for 30–45 min. Thereafter 0.9 ml of a RAC solution containing human albumin (BPL, Ellstree, UK), chloroquine (Sigma) in RPMI medium (Sigma) with final concentrations of 0.092% for albumin and 120 μM for chloroquine, respectively. Cells were washed once with PBS before addition of 1 ml of transfection solution and incubation for 4–5 h at 37°C in 5% CO_2 before removal of the transfection solution and addition of 2 ml of fresh complete medium. Transfected cells were harvested 48 h later, washed once with PBS and then incubated for 10 min at RT in 200 μl lysis buffer (10 mM sodium phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM EDTA pH 8.0, 1% Triton X-100 and 15% glycerol). Luciferase

activity was determined following addition of luciferase assay buffer (0.1 mM luciferin, 0.44 mM ATP in lysis buffer) using a luminometer (Berthold, Wildbad, Germany). Activity was corrected by protein concentrations of the samples, determined using the BCA Protein Assay Reagent.

Western blot analysis

1×10^5 cells per six-well were infected with the indicated MOIs in infection medium by incubation for 90 min at 37°C in 5% CO_2. The virus-containing medium was then diluted 1:5 with complete medium and cells maintained for 2 days in the incubator. Cytoplasmic and nuclear extracts were prepared from infected cells as previously described[30] and protein concentration determined by using a D_C protein assay (BioRad, Hercules, CA, USA). 30–60 μg of extract were resolved by 12–13% SDS-PAGE using High Range Rainbow protein size markers (Amersham Pharmacia, Piscataway, NJ, USA) before transfer to a nitrocellulose membrane (Gelman Sciences, Ann Arbor, MI, USA). The membrane was blocked with TBS/0.1% Tween20/5% milk powder for 1 h at RT. Primary antibodies were diluted in blocking buffer as follows: goat anti-NTR (Polyclonal Antibodies, Dyfed, UK): 1:2000; rabbit anti-LacI (Stratagene) 1:3000 and rabbit anti-p53 FL-393 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:500. Membranes were incubated with primary antibodies for 1 h at RT, washed extensively with TBS/0.1% Tween20 and incubated with secondary antibodies in TBS/0.1% Tween20/0.5% milk powder for 30 min at RT as following: donkey anti-rabbit 1:20 000 (Jackson ImmunoResearch, West Grove, PA, USA) and donkey anti-goat 1:7500 (Sigma). After extensive washing, enhanced chemiluminescence was carried out using the SuperSignal ULTRA detection system (Pierce) as described by the manufacturer. Autoradiographs were densitometrically scanned using a BioRad GS-690 scanner and Molecular Analyst software (version 1.4).

Nitroreductase quantitation by ELISA

Cytoplasmic extracts were prepared from infected cells by washing the cells with PBS and lysis in ice-cold hypotonic lysis buffer (10 mM Tris pH 7.5) for 45 min on ice. Extracts were cleared of debris by centrifugation at 13 000 r.p.m. for 2 min. For IPTG treatment, cells were incubated with 3 mM IPTG immediately following infection. Additional IPTG (final concentration of added IPTG = 1 mM) was added 24 and 48 h after infection. 96-well Maxisorp Assay Plates (Nunc-Immuno, Roskilde, Denmark) were coated in triplicate with 50 μl extract overnight. Wells were washed three times with PBS/0.5% Tween20 and then incubated with 100 μl PBS/0.5% Tween20 containing 1:2000 diluted primary goat anti-NTR antibody for 30 min at RT. Extracts were washed as above and then incubated with 1:5000 diluted secondary antibody in PBS/0.5% Tween20 for 30 min at RT. After three washes with PBS, wells were incubated with 100 μl of a solution prepared by mixing 1 ml of 1 mg/ml TMB (Sigma) in DMSO, 9 ml 0.05 M phosphate-citrate buffer (Sigma) and 2 μl 30% (v/v) H_2O_2. Wells were incubated for 15 min at RT and the reaction was then stopped with 25 μl 2 M H_2SO_4. Expression was calculated by reading the OD at 450 nm.

Animal studies

Subcutaneous SW480 tumour xenografts were generated in one flank of Balb/c nu/nu mice (Harlan, Bicester, UK) by injection of 2×10^6 logarithmic phase cells and monitored as previously described.[17] The virus preparation was directly injected into the tumour through the skin using a U-100 insulin syringe (Terumo, Leuven, Belgium) fitted with a fixed 27-gauge needle. CB1954 was prepared and administered as previously described.[17] Tail vein injection of virus (100 μl) into nude mice was carried out using a 1A syringe fitted with a fixed 27-gauge needle. CB1954 was administered beginning 48 h after injection as described for the xenograft study. Mice were weighed daily and monitored for signs of stress. Mice were killed humanely if mouse body weight was reduced by more than 20% or at the onset of any sign of severe stress. For NTR immunostaining of mouse livers, these were excised following death and processed as previously described.[17]

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